Lasers in Surgery and Medicine 26:48–57 (2000)

Long-Term Effectiveness of Photodynamic Therapy by Using a Hydrophilic Photosensitizer ATX-S10(Na) Against Experimental Choroidal Neovascularization in Rats

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Background and Objective: **We previously demonstrated that a hydrophilic photosensitizer ATX-S10 had a potent photodynamic effect. This study was designed to reveal the long-term effectiveness of photodynamic therapy (PDT) with this agent in occluding choroidal neovascularization (CNV) and its selectivity in the neovascular tissue.**
Study Design/Materials and Methods: **Experimental CNV was induced by intense photocoagulation in rat eyes. Immediately or 2 hours after intravenous injection of 8 mg/kg body weight of ATX-S10(Na), a *cis* isomer of ATX-S10, eyes were irradiated by a diode laser at the radiance of 3.25–65.3 J/cm² Vascular occlusion was identified by fundus photography, fluorescein angiography, and histology at 1, 3, 7, 14, and 28 days after PDT. As controls, non-neovascular eyes were subjected to PDT and similarly analyzed.**
Results: **By using the following treatment parameters, PDT with ATX-S10(Na) successfully occluded CNV without causing occlusion of retinal capillaries for 28 days; 7.4 and 19.6 J/cm² immediately after dye injection and 36.7 and 65.3 J/cm² 2 hours after injection. Although these conditions also caused occlusion of normal choriocapillaries and mild injuries of retinal vessels, retinal pigment epithelium, and photoreceptors at 1 day, retinal vessels and pigment epithelial cells recovered from damages by 28 days. No injuries were found in the inner retina.**
Conclusion: **In optimal treatment conditions, PDT with ATX-S10(Na) can induce long-term, selective occlusion of CNV without causing irreversible damages in the inner retina. Lasers Surg. Med. 26:48–57, 2000.** **© 2000 Wiley-Liss, Inc.**

Key words: ATX-S10(Na); choroidal neovascularization; photodynamic therapy; long-term effectiveness; retinal damage

INTRODUCTION

Laser photocoagulation has long been used in clinic as effective treatment modality for choroidal neovascularization (CNV) in age-related macular degeneration [1–3]. However, it often produces damage to the sensory retina, incomplete CNV closure, and frequent neovascular recurrence, resulting in a visual loss [3]. As for alternative therapeutic procedures such as sub-

Grant sponsor: Ministry of Health and Welfare; Grant number: Health Science Research Grants H10-018.

The authors have no financial interest in the present issue.

This article was presented in part at the Annual Meeting of the Association for Research in Vision and Ophthalmology in 1998.

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Accepted 29 September 1999

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macular surgery, radiation, and interferon administration, the efficacy remains unclear [4–9].

Photodynamic therapy (PDT), which was originally developed as a treatment modality for malignant tumors [10], has aroused great interest among ophthalmologists as a promising new method for CNV treatment [11,12], because of its potential for destroying vascular endothelial cells [13]. Clinical studies on PDT have been carried out by using liposomal benzoporphyrin (BPD) and tin-ethyl etiopurpurin (SnET2) as photosensitizers [14,15]. These two agents are lipid-soluble, so they need to be continuously infused into the vein. Recently, a water-soluble photosensitizer ATX-S10(Na) has been developed and can be administered as a bolus intravenous injection [16,17]. We previously revealed that PDT with this agent was effective for occlusion of experimental CNV and corneal neovascularization in rats, rabbits, and monkeys [18–20] and determined the optimal therapeutic parameters, i.e., dye dosage, irradiation dose, and time intervals between dye injection and laser irradiation, for the selective occlusion of neovascularization. Another problem in the PDT with liposomal BPD was recanalization and recurrence of neovascularization, which frequently occurred at the late periods after PDT [14,15]. We here examined the optimal conditions of PDT by using ATX-S10(Na) that produce long-term effectiveness with regard to selective occlusion of CNV and low incidence of recanalization or recurrence.

MATERIALS AND METHODS

Animals

Thirty-four eyes from 17 Brown-Norway rats (200–250 g body weight) were used. Animals were always treated in accordance with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

Induction of Experimental CNV

Rats were anesthetized by the intraperitoneal injection of 10 mg/kg body weight of Nembutal. The pupils were mydriatic with 0.5% tropicamide and 0.5% phenylephrine hydrochloride (Midrin P, Santen Co., Osaka, Japan). For topical anesthesia, 0.4% oxybuprocaine hydrochloride (Benoxil, Santen Co.) was used. Intense photocoagulation was induced at four sites in the fundus, sparing large-sized retinal vessels located near the optic disc, with an argon green laser (Coherent, CA; $\lambda = 514$ nm). A 100-μm spot was irradiated for 0.1 seconds with a power of 150 mW. At 20–25 days after photocoagulation, induction of CNV was confirmed by fundus photography and fluorescein angiography by using a fundus camera (Jenesis Kowa, Nagoya, Japan). Fluorescein angiography was conducted at various time intervals after intravenous injection of 0.2 ml of 5% fluorescein sodium.

Photodynamic Therapy

One day after confirming the presence of CNV, 8 mg/kg body weight of ATX-S10(Na) (Photochemical, Inc., Okayama, Japan) dissolved in water at the concentration of 10 mg/ml was injected into the tail vein. Immediately or 2 hours after dye injection, the site of CNV was irradiated by a 670-nm diode laser (Hamamatsu Photonics, Hamamatsu, Japan) as previously reported [18]. The spot-size was set large enough to cover the entire lesion of CNV. Irradiation at the retinal surface was calculated from the values at the corneal surface according to our previous study [18]. To evaluate the influence of radiant exposure on the photodynamic effect, radiant exposure was varied from 3.2 to 65.3 J/cm^2, by keeping the time of treatment constant (either 120 or 240 seconds) and changing irradiance. To assess the influence of irradiance on the photodynamic effect, irradiance was varied from 27.3 to 272 mW/cm^2, keeping the radiant exposure constant (10 J/cm^2 when irradiated immediately after injection and 50 J/cm^2 when irradiated 2 hours after injection) and changing the time of treatment. Table 1 summarizes the treatment parameters and numbers of tested lesions. As controls, two animals received either dye injection or laser irradiation alone.

For the analysis of photodynamic effect on the normal retina, laser irradiation was applied to the normal fundus by using the therapeutic parameters that had been demonstrated to induce selective occlusion of CNV. The treatment parameters and numbers of tested eyes are shown in Table 2.

Assessment of Therapeutic Efficacy

Therapeutic efficacy of PDT was evaluated at 1, 3, 7, 14, and 28 days after PDT based on fluorescein angiographic and histologic observations. For histology, at 28 days after PDT, eyes were enucleated under anesthesia. The CNV le-

TABLE 1. Photodynamic Therapy to Choroidal Neovascularization: Numbers of Tested Eyes and Treatment Parameters*

Irradiance (mW/cm^2)	Time (second)	Radiant exposures (J/cm^2)	No. of treated lesion (no. of rats used) Time intervals of irradiation after dye injection 0 h	2 h
27.3	120	3.27	10 (10)	nt
61.7	120	7.40	10 (10)	9 (9)
163.3	120	19.6	10 (10)	9 (9)
152.9	240	36.7	9 (9)	8 (8)
272	240	65.3	nt	8 (8)
27.3	366	10.0	3 (2)	nt
272	36	10.0	4 (2)	nt
27.3	1831	50.0	nt	3 (2)
272	183	50.0	nt	3 (2)

*nt, not tested.

TABLE 2. Photodynamic Therapy to Normal Retina: Numbers of Tested Eyes and Treatment Parameters*

Irradiance (mW/cm^2)	Time (second)	Radiant exposures (J/cm^2)	No. of treated lesion (no. of rats used) Time intervals of irradiation after dye injection 0 h	2 h
61.7	120	7.40	10 (5)	nt
163.3	120	19.6	10 (5)	nt
152.9	240	36.7	nt	10 (5)
272	240	65.3	nt	10 (5)

*nt, not tested.

sions were excised and fixed in Karnovsky's fixative overnight at 4°C. They were post-fixed in 2% OsO4 in 0.1 M phosphate buffer, pH 7.4, dehydrated in ethanol and embedded in Polybed (Polyscience, Inc., Warrington, PA). Semithin sections were stained with toluidine blue and observed by light microscopy. Thin sections were stained with uranyl acetate and lead citrate and observed under a JEM-1200EX electron microscope (JEOL, Tokyo, Japan).

RESULTS

Photodynamic Effect of ATX-S10(Na) on CNV

Before PDT, CNV induction was identified by fundus photography as subretinal whitish lesions and by fluorescein angiography as a network appearance in the early phase and fluorescein leakage in the late phase (Fig. 1A). After PDT, the CNV closure and retinal capillary injury were identified by the fluorescein angiographic findings of no fluorescein leakage and poor perfusion, respectively. The results of PDT with regard to the effectiveness and selectivity were divided into three categories: (1) "not effective," both CNV and retinal capillaries were patent; (2) "effective," CNV was closed, whereas retinal capillaries were open (Fig. 1B); and (3) "excessive," both CNV and retinal capillaries were closed (Fig. 2A). However, CNV lesions that received "effective" PDT often displayed reappearance of fluorescein leakage representative of recanalization within 28 days (Fig. 1C). Retinal capillaries that had closed after "excessive" PDT, as well, often underwent recanalization within 28 days (Fig. 2B).

The optimal radiant exposure for "effective" PDT was investigated in both conditions of immediately (Fig. 3A) and 2 hours (Fig. 3B) after dye injection. In the former condition, the radiant exposure of 3.27 J/cm^2 yielded the highest percentage (40%) of "effective" treatment at 3 days but showed the lowest value (10%) at 28 days (Fig. 3A). Furthermore, recanalization of CNV frequently (86%) occurred at 28 days. On the other hand, the radiant exposure of 7.4 and 19.6 J/cm^2 showed high percentages of "effective" treatment throughout the experimental periods (Fig. 3A). Although retinal capillaries were frequently (70% and 80%) closed at 3 days, they were gradually recanalized, thus leading to elevation of the percentages of "effective" treatment at 28 days. The radiant exposure of 36.7 J/cm^2 caused "excessive" treatment at any time period (Fig. 3A). In the condition of laser irradiation 2 hours after dye injec-

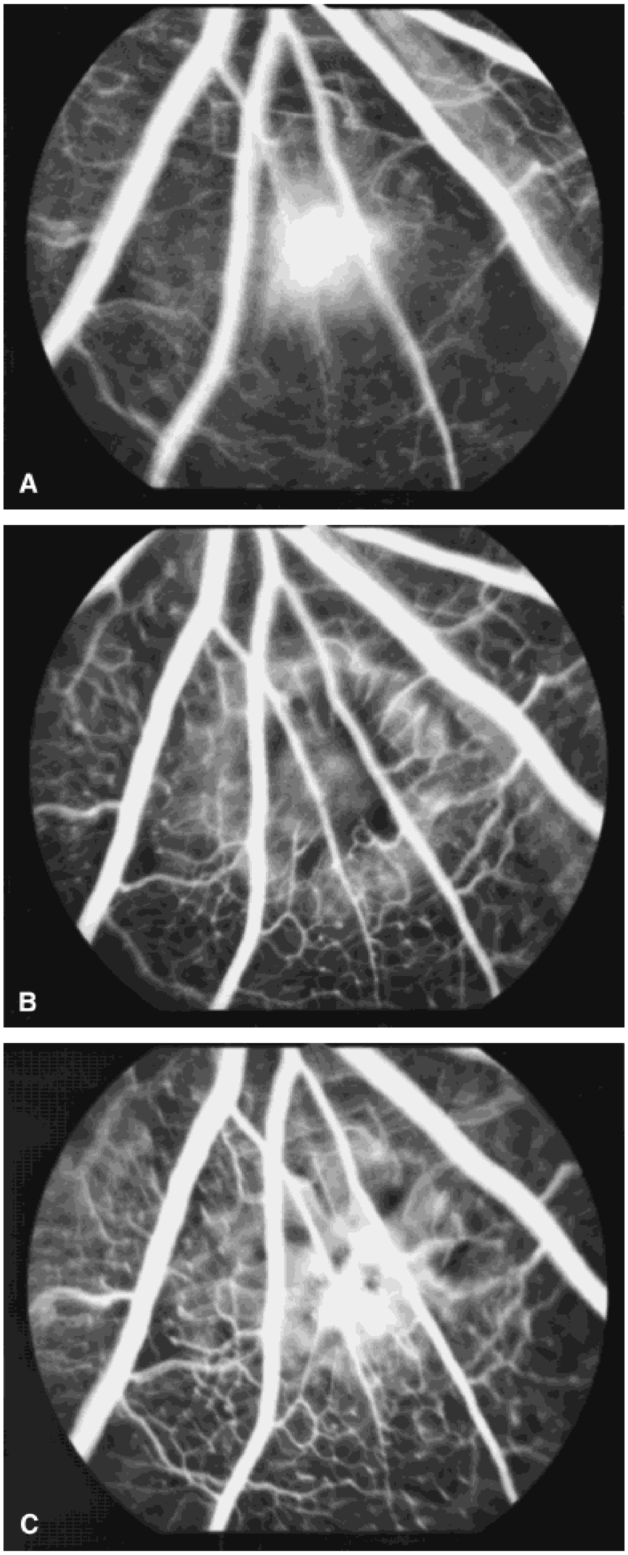

Fig. 1. Fluorescein angiograph of choroidal neovascularization (CNV) before (A), and 3 days (B) and 28 days (C) after photodynamic therapy. Irradiation was done 2 hours after ATX-S10(Na) injection with the radiant exposure of 7.40 J/cm^2. **A:** Fluorescein leakage is found in the lesion of CNV in the late phase of angiography. **B:** Fluorescein leakage is no longer seen. Retinal capillaries in the irradiated portion remain intact. **C:** Fluorescein leakage takes place in the neovascular lesion, indicating the recanalization.

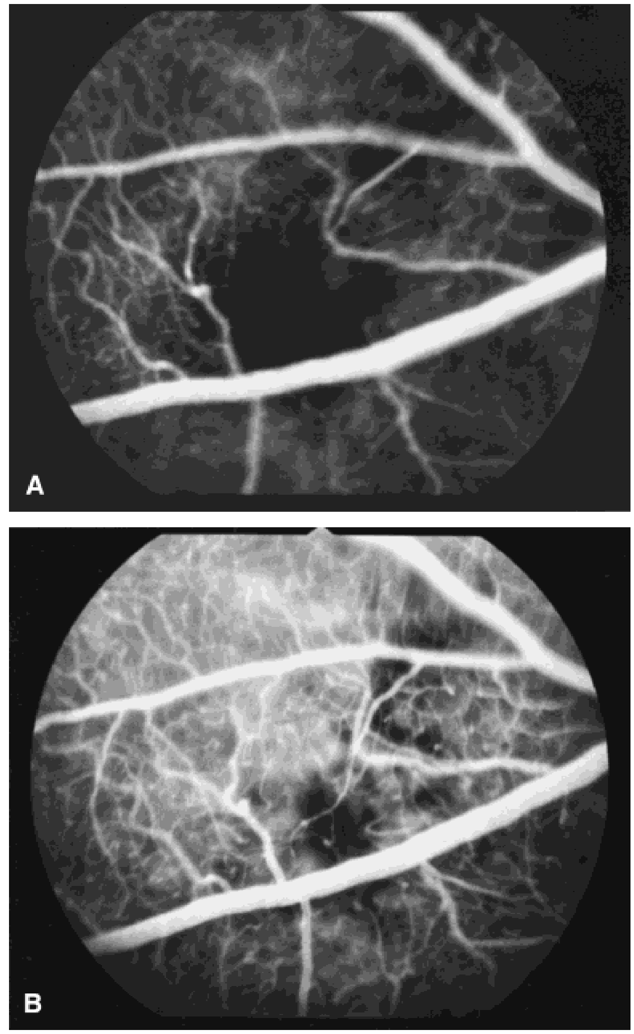

Fig. 2. Fluorescein angiograph of choroidal neovascularization (CNV) 3 days (A) and 28 days (B) after photodynamic therapy. Irradiation was done 2 hours after ATX-S10(Na) injection with the radiant exposure of 36.7 J/cm^2. **A:** Both CNV and retinal capillaries are occluded, giving rise to a fluorescein defect. **B:** There is no fluorescein leakage in the neovascular lesion. Retinal capillaries are well perfused.

tion, photodynamic effects were generally milder than in the condition of irradiation immediately after injection. The radiant exposure of 36.7 and 65.3 J/cm^2 gave higher percentages (63% and 75%, respectively) of "effective" treatment than that of 7.4 and 19.6 J/cm^2 (22% and 44%, respectively) at 28 days (Fig. 3B).

We further examined the effectiveness of PDT by different irradiances, keeping radiant exposure constant. In both conditions of the radiant exposure of 10 J/cm^2 irradiated immediately after injection (Fig. 4) and 50 J/cm^2 2 hours after injection, CNV closure and retinal capillary damages

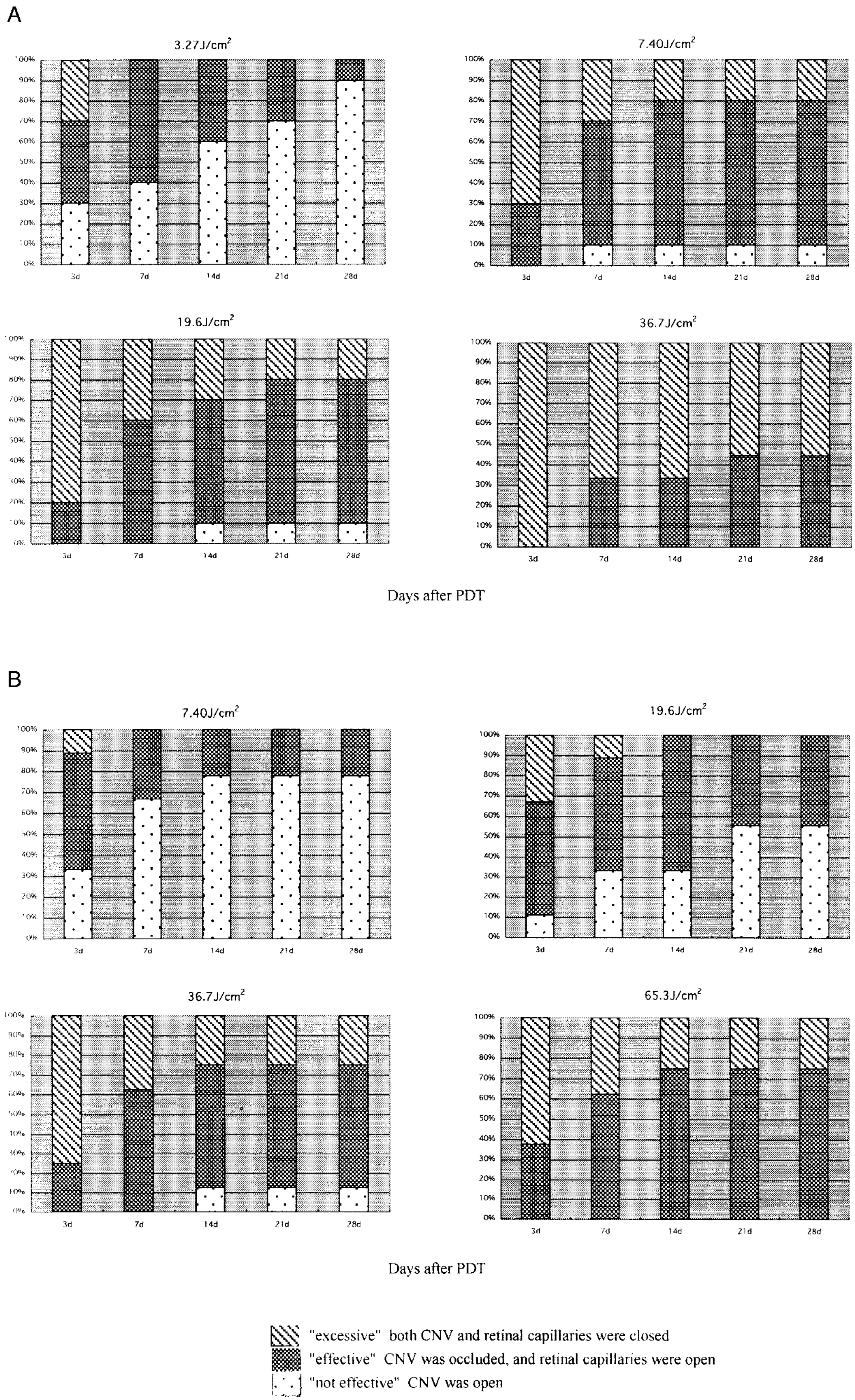

Fig. 3. Time course analysis of the proportions of "not effective," "effective," and "excessive" treatment by photodynamic therapy performed immediately (**A**) and 2 hours (**B**) after dye injection. Radiant exposure varied from 3.27 to 65.3 J/cm^2. Results were evaluated by fluorescein angiography and classified into three groups: (1) "not effective", choroidal neovascularization (CNV) was open; (2) "effective" CNV was occluded, and retinal capillaries were open; and (3) "excessive", both CNV and retinal capillaries were closed.

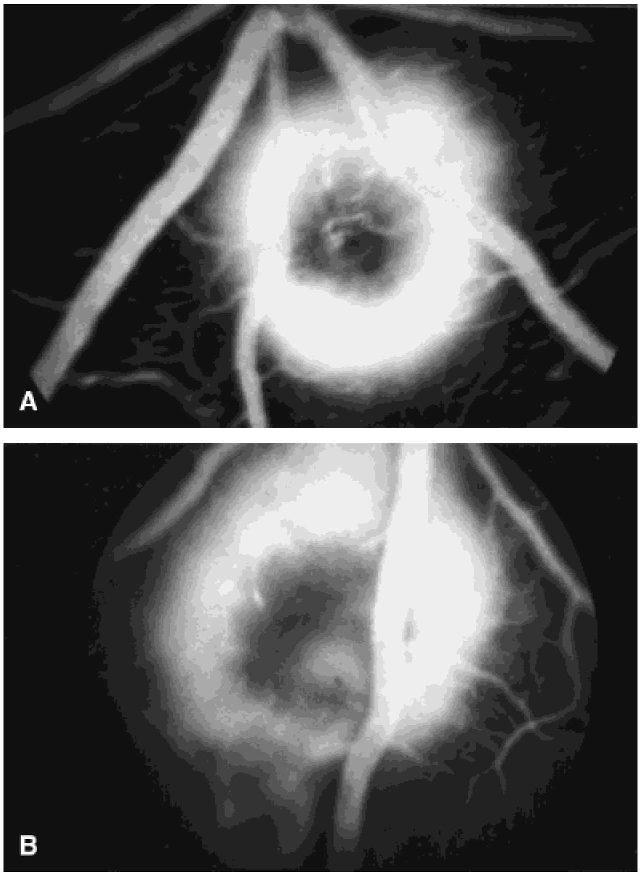

Fig. 4. Fluorescein angiograph of choroidal neovascularization (CNV) treated with different irradiances (**A:** 27.3 mW/cm^2; **B:** 272 mW/cm^2), keeping the radiant exposure constant (10 J/cm^2), at 1 day after photodynamic therapy. CNV was similarly closed in both lesions. The areas of damaged retinal pigment epithelium and occlusion of choriocapillaries are also similar.

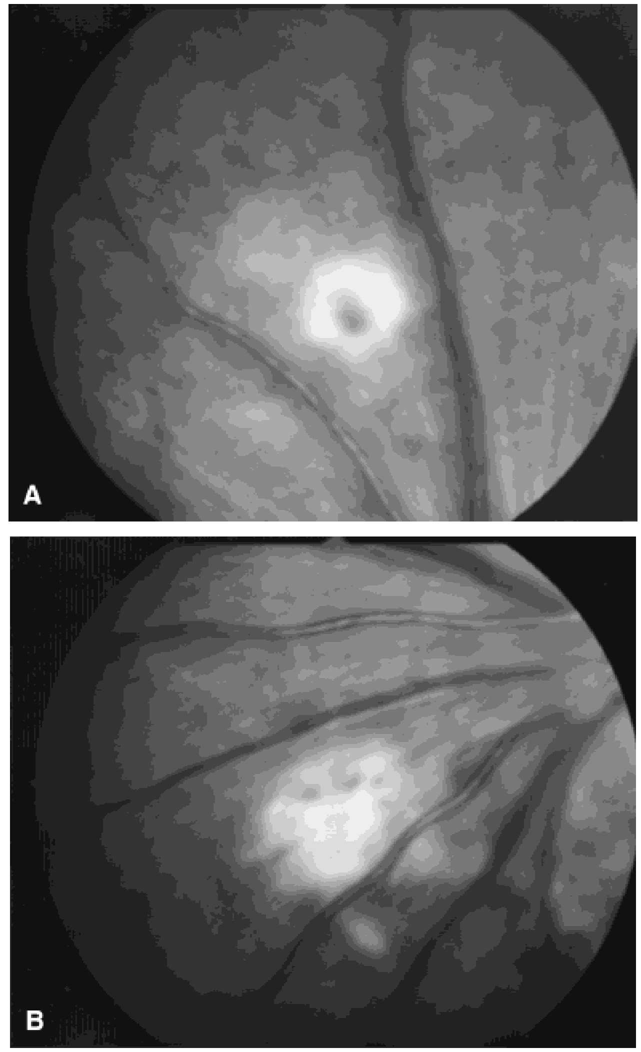

Fig. 5. Fundus photographs of "effective" (A) and "excessive" (B) treatment by photodynamic therapy at 28 days. Irradiation was done 2 hours after ATX-S10(Na) injection. **A:** The radiant exposure of 36.7 J/cm^2. There is subretinal fibrous tissue and pigmentation at the center and margin, respectively, of the irradiation field. **B:** The radiant exposure of 65.3 J/cm^2. Retinal pigment epithelium is atrophic around the subretinal fibrous tissue.

were similar between the irradiance of 27.3 mW/cm^2 (Fig. 4A) and 272 mW/cm^2 (Fig. 4B).

When lesions were "effectively" treated by PDT, no exudative retinal detachment occurred as demonstrated by fundus photography, although there remained subretinal whitish proliferative tissue and pigmentation as the consequence of photocoagulation (Fig. 5A). On the other hand, when they were "excessively" treated, retinal pigment epithelium in the irradiated area became atrophic (Fig. 5B).

Light and electron microscopy confirmed the complete occlusion of CNV in the subretinal proliferative tissue at 28 days after "effective" PDT (Fig. 6A,B). Basal lamina-like structures remained around the remnant of CNV (Fig. 6B). Retinal pigment epithelial cells and surrounding fibroblasts showed prominent proliferation.

Photodynamic Effects of ATX-S10(Na) on the Normal Retina

Because the present neovascularization model included photocoagulation-induced retinal and choroidal damages, to evaluate the injuring effect of PDT on the normal tissue, we irradiated non-neovascularized eyes with the parameters that gave "effective" CNV occlusion, i.e., 7.4 and 19.6 J/cm^2 just after dye injection and 36.7 and 65.3 J/cm^2 2 hours after injection. Results were similar among these parameters. At 1 day after PDT, the irradiation portion of the retina looked

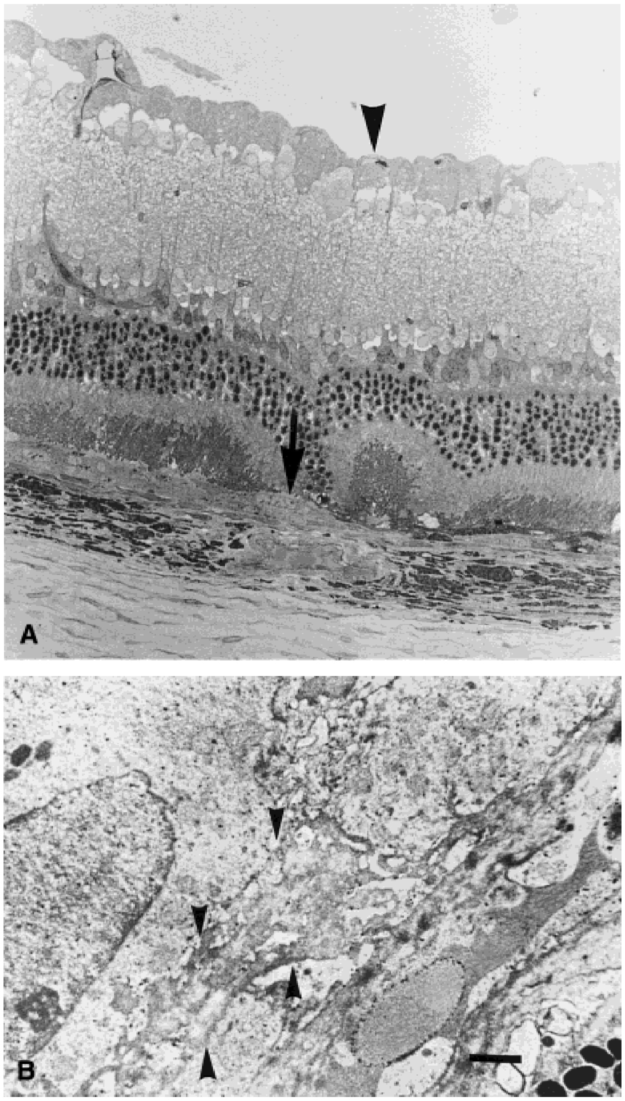

Fig. 6. Light micrograph (A) and electron micrograph (B) of "effective" treatment of choroidal neovascularization (CNV) by photodynamic therapy (PDT) at 28 days. PDT was done with the radiant exposure of 7.4 J/cm^2 just after ATX-S10(Na) injection. **A:** Neovascularization in the subretinal proliferative tissue (arrow) is occluded, whereas retinal capillaries are intact (arrowheads). Original magnification, ×66. **B:** Neovascularization is diminished in the subretinal proliferative tissue, but basal lamina-like structures (arrowheads) remain. Scale bar = 1 μm.

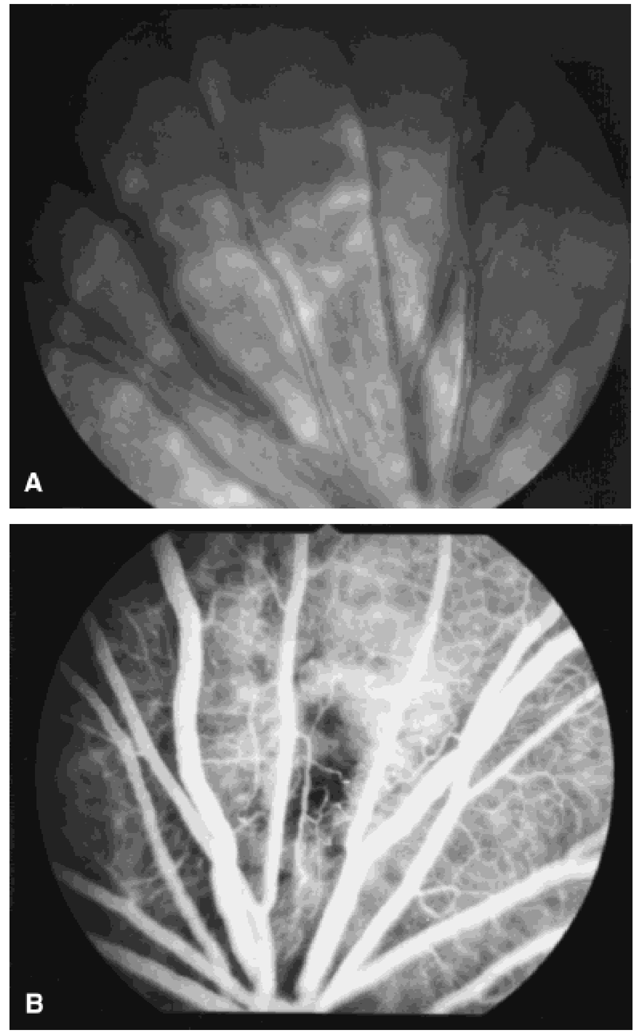

Fig. 7. Fundus photograph (A) and fluorescein angiograph (B) of the non-neovascular funds at 28 days after PDT. Irradiation was done at the radiant exposure of 7.4 J/cm^2 just after dye injection. **A:** Retinal whitening disappears, leaving pigmentation. The boundary between treated and nontreated area becomes obscure. **B:** There is only slight hypofluorescence at the center of irradiation field. Fluorescein leakage is not seen.

whitish by fundus photography and displayed by fluorescence angiography central hypofluorescence indicative of choriocapillary occlusion in the early phase and marginal fluorescein leakage indicative of disruption of retinal pigment epithelial layer in the late phase. Retinal capillary occlusion was observed in three of 10 lesions, whereas retinal arteriole/venule occlusion was not detected. At 3 days, retinal capillary occlusion decreased in incidence to one of eight lesions. Fluorescein leakage also decreased. At 7 days, all the retinal capillaries were recanalized. At 14 and 28 days, retinal whitening disappeared by fundus photography, making the boundary of lesions obscure and leaving pigmentation (Fig. 7A). By fluorescein angiography, fluorescein leakage was no longer seen, although hypofluorescence in the early phase remained (Fig. 7B).

Light microscopically, choriocapillaries remained occluded from day 1 (Fig. 8A) to day 28 (Fig. 8B), whereas retinal capillaries were patent at 28 days. Electron microscopically, the lumens

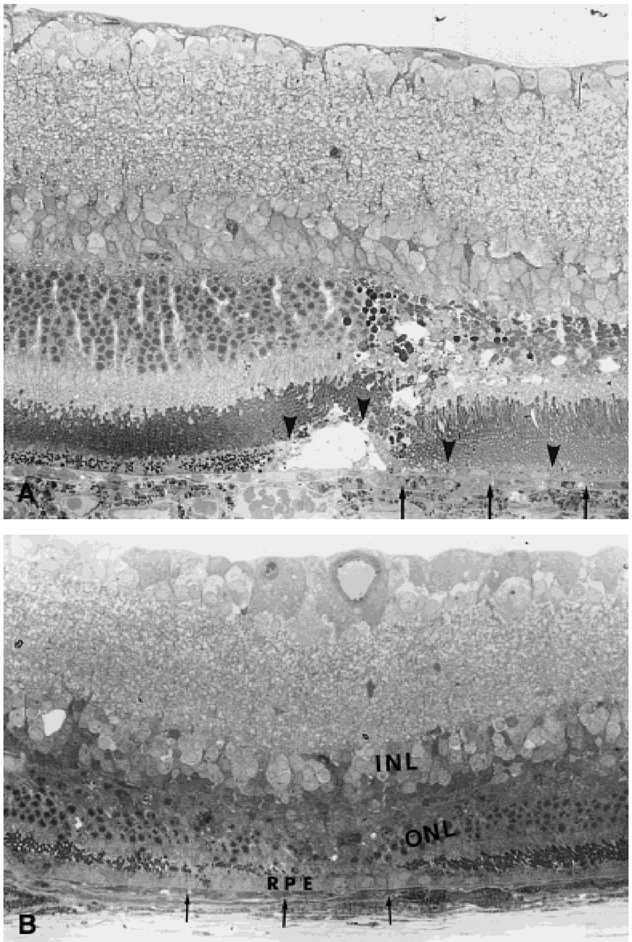

Fig. 8. Light micrographs of non-neovascular fundus at 1 day (A) and 28 days (B) after photodynamic therapy. Irradiation was done at the radiant exposure of 7.4 J/cm^2 immediately after dye injection. **A:** Choriocapillaries at the center of irradiated area are occluded (arrows). Retinal pigment epithelial cells undergo necrosis in a wider area (arrowheads) than choriocapillary closure. **B:** Although the inner nuclear layer of the retina (INL) shows no alterations, the outer nuclear layer (ONL) shows a decreased number of cells with disappearance of outer and inner segments of photoreceptor cells. Retinal pigment epithelial cells (RPE) are partially stratified. Choriocapillaries are occluded in the irradiation area (arrows), but retinal vessels and choroidal arteries and veins are intact. Original magnification, ×100.

of retinal capillaries were often occupied with thrombus at 1 day, but no necrotic changes were detected in the endothelial cells. Retinal and choroidal arterioles/venules showed no morphologic changes. Although retinal pigment epithelial cells and photoreceptor cells were injured at 1 day, the former cells were repaired by day 7 through regeneration, but the latter cells remained damaged until 28 days. The inner nuclear layer of the retina was not injured.

DISCUSSION

We previously reported that PDT with ATX-S10 effectively occluded CNV without closing the retinal vessels one day after irradiation in the following conditions, i.e., the radiance exposure of 7.4 J/cm^2 immediately after injection of 16 mg/kg body weight ATX-S10 and 22.0 J/cm^2 2–4 hours after injection [18]. The present study has demonstrated that this procedure yields a long-term, selective occlusion of CNV until 28 days without causing irreversible damages to the inner retina. (We used here 8 mg/kg body weight ATX-S10[Na], because ATX-S10[Na] contains only a *cis*-isomer and has more potent photochemical effects than conventional ATX-S10 [21]).

The optimal parameters of irradiation for the long-term occlusion of CNV were (1) radiant exposure of 7.4 and 19.6 J/cm^2 immediately after dye injection, and (2) 36.7 and 65.3 J/cm^2 2 hours after injection. Although these conditions of PDT also induced retinal capillary occlusion to some extent at 1 day, the occlusion was reversible and recanalization developed until 7 days, thus increasing the rate of "effective" treatment at 28 days. Although the rate of "effective" treatment was higher in radiant exposure of 3.27 J/cm^2 compared with these optimal conditions at 1 day, many CNV underwent recanalization at 28 days. The mechanism of recanalization after PDT is not well understood. The present histologic finding that thrombus formation but not endothelial destruction was responsible for retinal capillary closure suggests that recanalization may be the phenomenon of recovery of the blood flow from impairment. In our previous experiment that used the monkey [19], retinal capillaries were not occluded in the optimal conditions of PDT which induced CNV closure. Therefore, there remains the possibility that dye dosages smaller than 8 mg/kg body weight may induce more selective occlusion of CNV in rats.

It has been demonstrated for other photosensitizers that lower irradiance improves the PDT effect even in the same radiant exposures [22–24]. In particular, Sitnik and Henderson [24] suggested that photodynamic therapy carried out at low irradiance may enhance tumor response and selectivity. However, in the present study on the PDT with ATX-S10(Na), CNV was similarly occluded by different irradiances at the same radiant exposures.

In the PDT-treated non-neovascular eyes, although the choriocapillaries, retinal pigment epi-

thelial cells, and photoreceptor cells were damaged, the inner nuclear layer, ganglion cell layer, nerve fiber layer, and choroidal arteries/veins were free from injuries, indicating that PDT induced much less damage to the normal tissue compared with laser photocoagulation, which affects all layers of the retina [25,26]. Our previous study demonstrated that intravenously injected ATX-S10 distributed in the choriocapillaries and retinal pigment epithelial cells but not in the photoreceptor cells [18]. By taking this data into account, injuries in the choriocapillaries and retinal pigment epithelial cells are considered to result from the primary action of PDT, whereas those in photoreceptor cells might be secondary to primary injuries. Consistent with this view, injuries of photoreceptor cells did not appear soon after PDT [18] but gradually advanced at later than 1 day as shown here. Furthermore, different from the injuries of photoreceptor cells that persisted until 28 days, those of retinal pigment epithelial cells were repaired by means of epithelial regeneration and, as the consequence of recovery of barrier function, edema in the outer layer of retina was improved at 28 days.

In conclusion, PDT with ATX-S10(Na), with carefully optimized delivery and treatment parameters, can be shown to induce the long-term, selective occlusion of CNV without causing irreversible damages to retinal vessels and inner layer of the retina. Considering the inevitable injury of photoreceptor cells by PDT, unnecessarily broad irradiation should be avoided.

ACKNOWLEDGMENTS

The authors thank Isao Sakata, PhD, Photochemical, Inc., Okayama, and Ryosuke Machida, PhD, Wyeth-Lederle Japan, Tokyo, for providing ATX-S10(Na) and Mr. Toru Hirano, Hamamatsu Photonics, Inc., Hamamatsu, for providing the diode laser.

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